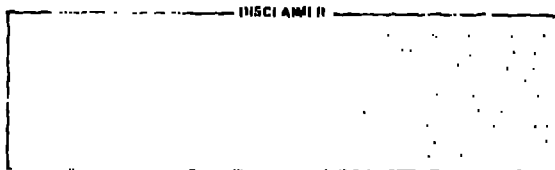**TITLE:** Genes Coding for Metal Induced Synthesis of RNA Sequences Are Differentially Amplified and Regulated in Mammalian Cells

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Genes Coding for Metal Induced Synthesis
of RNA Sequences Are Differentially Amplified
and Regulated in Mammalian Cells

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I. ABSTRACT

We have isolated three variant cell lines which survive cadmium (Cd^{++}) concentrations 10-200 fold greater than that which kills parental Chinese hamster cells (line CHO). Cd^{++} treatment of the variants induces the synthesis of a highly abundant poly A^+ RNA class which directs the synthesis of metallothionein in a cell free translation system. Hybridization of cDNA complementary to these inducible, highly abundant RNA sequences ($cDNA_a$) with RNA from variant cells showed that: (i) the induced abundant class has a total complexity of $\sim$2000 NT; (ii) Cd^{++} induction increases the cellular concentration of these sequences $\sim$2000 fold above preinduction levels in each of the variants; (iii) most, if not all, of these sequences are expressed constitutively in uninduced cells. Cd^{++} induction of sensitive CHO cells increases the cellular concentration of only a subset of the sequences inducible in resistant cells and then only to a level 100 fold higher than in uninduced cells; the remainder of the sequences could not be induced to a measureable extent. In addition, only $\sim$50% of the sequences are constitutively expressed at measureable levels in uninduced CHO cells. Hybridization of $cDNA_a$ with genomic DNA from the three resistant variants showed that genes coding for the induction specific RNA sequences are amplified $\sim$10 fold in Cd^r20F4 cells, $\sim$4 fold in Cd^r3C79 cells, and unamplified in Cd^r2C10 cells relative to CHO. While sensitive CHO cells can tolerate only 0.2μM Cd^{++}, Cd^r30F9, Cd^r20F4, and Cd^r2C10 cells are resistant to 40μM, 26μM, and 2μM Cd^{++} respectively. Thus, gene amplification alone cannot be responsible for the observed resistance of the variant cell lines.

II. INTRODUCTION

Cadmium is a toxic metal that persists in the environment, and the level of Cd^{++} to which individuals in industrialized nations have been exposed has already resulted in _in vivo_ body burdens less than an order of magnitude lower than that known to produce overt toxicity (1). Although the mechanism(s) by which a cell or organism ameliorates the cytotoxic effect of cadmium is not known, it is thought that the synthesis of metallothioneins (MT), small metal binding proteins, may play an important role by sequestering Cd^{++} in a non-toxic form (2-3).

In order to define the role(s) played by cellular processes in cadmium detoxification, the molecular events associated with cadmium exposure have been examined in Cd^{++} resistant and Cd^{++} sensitive Chinese hamster cells. Three cadmium resistant variants have been selected by stepwise culture in increasing Cd^{++} concentrations. Compared to the parental cell (line CHO), each of the resistant variants (i) has a higher cadmium toxic threshold, (ii) can synthesize more inducible MT and (iii) can accumulate more inducible translatable MT mRNA (4-5). While these results are consistent with a role for MT in the acquisition of a cadmium resistant phenotype, they reveal very little of the underlying molecular mechanisms responsible for Cd^{++} resistance. We have initiated studies designed to probe cellular responses to Cd^{++} treatment and report here that Cd^{++} treatment of the resistant variants induces the synthesis of a highly abundant poly A^+ RNA class, a major portion of which is metallothionein mRNA. Not only is this RNA class differentially regulated in resistant variant cells but the structural genes encoding these inducible RNAs are differentially amplified in the resistant variants. However, there is not a direct correlation between the degree of gene amplification and resistance. Although the acquisition of Cd^{++} resistance may be a considerably more complicated phenomenon than originally expected, these resistant variants provide models useful for the study of both regulation of inducible gene function and the factors responsible for Cd^{++} detoxification.

III. METHODS

The conditions of cell culture, derivation of resistant variants, and properties of the three resistant variant Chinese hamster cell lines $Cd^r 30F9$, $Cd^r 20F4$, and $Cd^r 2C10$ have been described elsewhere (3,5). In none of the variants derived from the parental CHO cell was cadmium resistance due to a failure of cells to transport cadmium from the

extracellular medium. In each of the experiments reported
here, Cd^{++} induction conditions were those previously shown to
result in maximum production of translatable metallothionein
mRNA: Cd^r30F9 cells - 40_μM $CdCl_2$ for 4h; Cd^r20F4 cells - 20μM
$CdCl_2$ for 4h; Cd^r2C10 cells - 2_μM $CdCl_2$ for 8 h; CHO cells -
2_μM $CdCl_2$ for 11 h (5).

We have previously described the conditions for poly A^+
RNA extraction, synthesis of ^{3}H labeled cDNA ($\sim$7x10^7 cpm/μg),
cDNA-poly A^+ RNA hybridization, and the S1 nuclease assay for
cDNA-RNA hybrid formation (5). All values of Rot and Cot were
corrected to the standard salt concentration (7).

A tracer ($cDNA_a$) complementary to Cd^{++} inducible, highly
abundant poly A^+ RNA was prepared by reacting cDNA copied from
induced Cd^r20F4 poly A^+ RNA to an ERot of 210 with a 1000 fold
mass excess of poly A^+ RNA from uninduced CHO cells. The
unreacted fraction was isolated from hydroxylapatite (HAP) and
then reacted with a 10,000 fold mass excess of poly A^+RNA from
induced Cd^r20F4 cells to ERot of 0.07. The double strand
fraction was isolated from HAP, hydrolyzed with 0.3N NaOH
(18h, 37°), dialyzed, and precipitated with ethanol and tRNA
carrier.

Genomic DNA was isolated from nuclei of the same cells
from which the cytoplasmic poly A^+ RNA was obtained. Nuclei
were prepared as previously described (6), and DNA was
isolated by the method of Kedes _et al_. (8) winding the DNA out
of ethanol at the final step. The DNA was sheared by
homogenization to a 300 nucleotide (NT) double strand length.
For hybridization, aliquots were prepared which contained
600 μg genomic DNA, 7 μg ^{14}C-labeled genomic DNA (cells grown
in 0.0075 μCi/Ml ^{14}C-thymidine for 3 cell doublings), and 13
pg of ^{3}H-cDNA$_a$. Samples were melted and annealed at 68° in 10
mM Tris (pH 7.5) - 1.5M NaCl - 0.2% sodium dodecyl sulfate -
2mM EDTA, and hybrid formation was assayed by HAP
chromatography.

IV. RESULTS

We recently showed that the poly A^+ RNA from Cd^{++} induced
Cd^r20F4 cells contains a highly abundant RNA class
undetectable in uninduced CHO cells (9). We have exploited
this difference in abundance to isolate from total induced
Cd^r20F4 cDNA a tracer ($cDNA_a$) complementary to induction
specific RNA sequences (see "Methods" for additional detail).
A comparison of the hybridization to induced Cd^r20F4 RNA of
$cDNA_a$ and total Cd^r20F4 cDNA is shown in Fig. 1A. The $cDNA_a$
hybridized, with an ERot$_{1/2}$ of 0.04, to the most abundant RNA
class which comprises $\sim$5% of the total induced Cd^r20F4 poly A^+
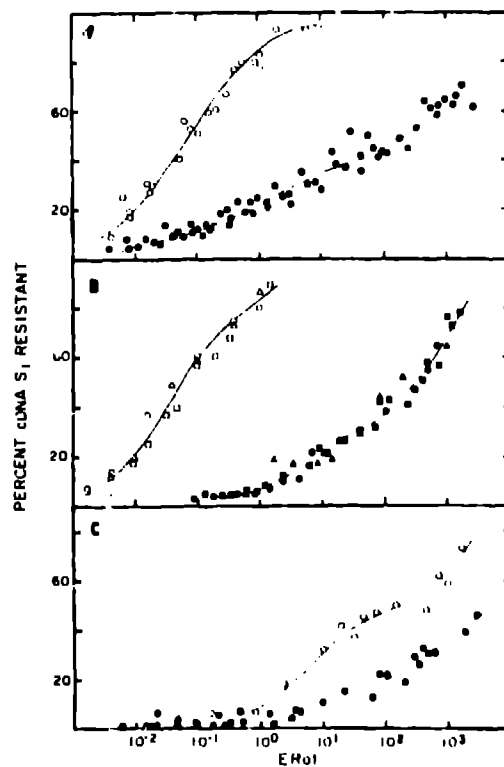RNA. Comparison of the data in Fig. 1A with that

Figure 1. Hybridization of cDNA to poly A^+ RNA. (A) Hybridization to Cd^{++} induced Cd^r20F4 RNA of total Cd^r20F4 cDNA (closed circles) and $cDNA_a$ (open circles). (B) Hybridization of $cDNA_a$ to RNA from Cd^r30F9 (circles), Cd^r20F4 (triangles), and Cd^r2C10 (squares) which had been either Cd^{++} induced (open symbols) or not induced (closed symbols) prior to RNA extraction. (C) Hybridization of cDNAa to RNA from Cd^{++} induced (open circles) or uninduced (closed circles) CHO cells.

measured earlier (6) for the hybridization of the kinetic standard chicken globin cDNA with its template ($ERot_{1/2}$ of 0.0018, 1820NT complexity) indicates that the total complexity of the induced class is ~2000NT. It will be noted that, at an ERot of 1, more than 80% of $cDNA_a$ is hybridized to induced Cd^r20F4 RNA while less than 4% is hybridized to uninduced CHO RNA (Fig. 1C) and that the concentration of RNA sequences complementary to $cDNA_a$ is at least 10,000 fold higher in induced Cd^r20F4 than uninduced CHO RNA. Furthermore, the shape of the $cDNA_a$-induced Cd^r20F4 RNA hybridization curve and its span of ERot indicate that the RNA sequences complementary to $cDNA_a$ are present at nearly equal concentrations. We have cloned and determined the nucleotide sequence of DNA molecules

complementary to $cDNA_a$. As will be reported elsewhere, the nucleotide sequence of one of the cloned DNAs is consistent with that predicted from the published amino acid sequence of mammalian metallothionein from other species (2). The extent to which the synthesis of RNA sequences complementary to $cDNA_a$ could be induced by cadmium was measured in each of the three cadmium resistant variants. Each cell type was exposed to cadmium under conditions known to induce the maximal synthesis of translatable metallothionein mRNA. As illustrated in Fig. 1B, there is little difference among the three resistant variants in either the constitutive level or the maximal level to which the RNA sequences complementary to $cDNA_a$ could be induced by cadmium treatment. Comparison of the $ERot_{1/2}$ of the $cDNA_a$ reactions with RNA from induced and uninduced cells indicates that cadmium treatment induces an ⋰2000 fold increase in the concentration of these sequences. The curve defining the reaction of $cDNA_a$ with RNA from uninduced cells (Fig. 1B) appears to show some biphasic character. If the biphasic character is real, the $ERot_{1/2}$ of the total reaction would slightly overestimate the relative constitutive concentration of the majority of the RNA sequences.

The reaction of $cDNA_a$ with RNA from cadmium induced and uninduced sensitive CHO cells is shown in Fig. 1C. In contrast to the cadmium resistant variant cells (Fig. 1B), the curve defining the reaction of $cDNA_a$ with induced CHO RNA is clearly biphasic. Only ⋰50% of the sequences complementary to $cDNA_a$ could be induced to an appreciable extent and then only to a maximum of 100 fold higher than the constitutive concentration. The remainder of the sequences hybridized with kinetics similar to that of the least abundant RNA (see Fig. 1A). It should be noted that the concentration of the subset of sequences induced in CHO is still < 1% that in the induced variant cells. Compared to the resistant variants, the constitutive concentration of these sequences in CHO is at least 10 fold lower at the point where hybridizations were terminated due to thermal instability of the RNA.

One way by which the resistant variant cells could attain an increased capacity for the synthesis of induction specific RNA sequences is by amplification of their respective structural genes. We tested for this possibility by annealing $cDNA_a$ to genomic DNA isolated from nuclei of each of the cell types. To be absolutely certain that the results could not be compromised by different rates of annealing of driver DNA, each hybridization mixture contained, as an internal standard, C-labeled genomic DNA; thus, the rates of annealing of both driver genomic DNA and $cDNA_a$ were assayed simultaneously. As shown in Fig. 2A, the rates of hybridization of genomic DNA from each of the cell types were experimentally
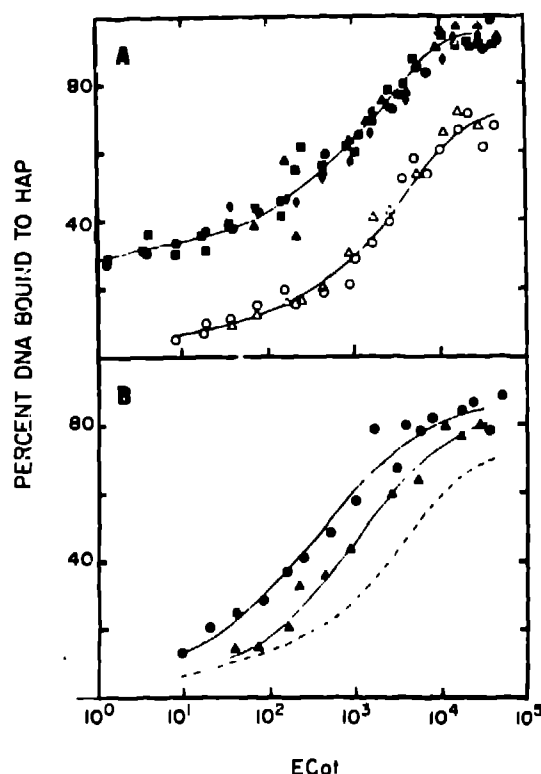
Figure 2. Amplification of genes encoding induction specific
RNA sequences. (A) Closed symbols represent annealing of
^{14}C-labeled genomic DNA to driver DNA isolated from Cd^{r}30F9
(circles), Cd^{r}20F4 (squares), Cd^{r}2C10 (triangles), and CHO
cells (diamonds). Open symbols represent annealing of cDNA$_a$
to genomic DNA from Cd^{r}2C10 (circles) and CHO cells
(triangles). (B) cDNA$_a$ was annealed to genomic DNA from
Cd^{r}20F4 (circles) and Cd^{r}30F9 cells (triangles). For
comparison, the dashed line shows the annealing of cDNA$_a$ to
DNA from Cd^{r}2C10 and CHO cells and is reproduced from panel A.

indistinguishable. cDNA$_a$ was hybridized to genomic DNA from
CHO, Cd^{r}2C10 (Fig. 2A), Cd^{r}20F4, and Cd^{r}30F9 cells (Fig. 2B).
The kinetics of reaction of cDNA$_a$ to CHO DNA and Cd^{r}2C10 DNA
were experimentally indistinguishable and were similar to that
of the single copy component of genomic DNA (Fig. 2A).
However, cDNA$_a$ reacted with Cd^{r}20F4 DNA and Cd^{r}30F9 DNA 10
fold and 4 fold faster, respectively, relative to CHO DNA
(Fig. 2B). These data show that, relative to CHO, the
structural genes coding for induction specific RNA sequences
are amplified 10 fold and 4 fold, respectively, in the genomes
of Cd^{r}20F4 and Cd^{r}30F9 and are unamplified in the genome of
Cd^{r}2C10. Since cDNA$_a$ annealed to CHO DNA with kinetics
similar to that of single copy DNA, the respective structural

genes must be present at no more than a few copies per genome
in cadmium sensitive CHO cells from which the cadmium
resistant variants were derived. Gene amplification is likely
not the result of formation of high heteroploid cells during
variant selection since the total DNA content of resistant
variant cells is the same as that of parental CHO cells based
on flow microfluorometric DNA analysis (data not shown).

V. DISCUSSION

Chronic exposure of sensitive cells to increasing
concentrations of particular toxic agents can result in the
acquisition of specific resistant phenotypes. Alt et al. (10)
and Wahl et al. (11) have shown that mammalian cells resistant
to methotrexate and N-(phosphonacetyl)-2-aspartate could be
derived by continuous culture in progressively increasing
concentrations of the drugs. In each case, resistance was
accompanied by (i) an increased production of the enzyme which
ameliorated the cytotoxic effects of the drug, (ii) an
increased production of the mRNA coding for the enzyme, and
(iii) amplification of the structural gene encoding the enzyme
to an extent sufficient to completely account for the
increased production of the respective mRNA. In some
respects, the scenario developed for the acquisition of a
cadmium resistant phenotype displayed by the Chinese hamster
variants studied here is similar to that outlined above for
acquisition of resistance to methotrexate and
N-(phosphonacetyl)-2-aspartate. In each case, the cadmium
resistant variant cell can synthesize more of the protein,
metallothionein, putatively associated with amelioration of
cadmium cytotoxicity (5) and can accumulate a minimum of 100
fold higher concentrations of poly A^+ RNA sequences (which
include the metallothionein mRNAs) induced by Cd^{++} treatment
(Fig. 1B) when compared to the sensitive parental CHO cell
(Fig. 1C); in two of the three resistant variants, the
structural genes encoding the induction specific RNAs have
been amplified.

However, for each of the properties the cadmium resistant
variants share with methotrexate and N-(phosphonacetyl)-
2-asparatate resistant variants, there are other important
properties that are quite different. These include the
observations that: (i) each of the cadmium resistant variants
synthesize approximately the same amount of induction specific
sequences when maximally induced (Fig. 1B); (ii) while the
induced concentration of these sequences in each of the
resistant variants is ∿2000 fold higher than the constitutive
level, the structural genes encoding these RNA sequences are
not amplified in Cd^r2C10 (Fig. 2A); (iii) differences in the

extent of gene amplification, 10 fold and 4 fold, in Cd^r2OF4 and Cd^r3OF9 cells, respsectively, are not accompanied by similar differences in the ability to synthesize Cd^{++} induced RNA sequences or in resistances to Cd^{++}; (iiii) although sensitive CHO and resistant Cd^r2C10 cells each have the same number of structural genes, CHO cells can synthesize only a subset of the induction specific RNAs (Fig. 1C) and then to a level <1% of that in Cd^r2C10 cells.

Since sensitive parental CHO cells can tolerate <0.2μM Cd^{++} while the independently derived Cd^r3OF9, Cd^r2OF4, and Cd^r2C10 cells are resistant to 40μM, 26μM, and 2μM Cd^{++}, respectively, it would appear that neither gene amplification nor the ability to synthesize induction specific RNAs is directly responsible for the acquisition of a Cd^{++} resistant phenotype. Nonetheless, this should not be interpreted to suggest that either of these parameters are unimportant and play no role in resistance. Certainly each of the resistant variants is more proficient than CHO in the synthesis of both metallothionein and induction specific RNA sequences after cadmium treatment (Fig. 1B). Further, the induction specific RNA sequences are differentially regulated in the resistant variants and sensitive CHO (Fig. 1C). It may be that other factors also contribute to heavy metal detoxification and resistance (5, 12-13). Each of these factors may be necessary but not alone sufficient for acquisition of resistance. Resolution of the role played by each of the factors will be an important step in understanding heavy metal detoxification.

VI. ACKNOWLEDGMENTS

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